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Major Article

Prevalence and distribution of *Angiostrongylus cantonensis* (Nematoda, Angiostrongylidae) in *Achatina fulica* (Mollusca, Gastropoda) in Baixada Santista, São Paulo, Brazil

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Abstract

Introduction: *Angiostrongylus cantonensis* causes eosinophilic meningoencephalitis in humans. Worldwide expansion of this nematode is linked to the dispersion of their hosts. This study aimed to determine the prevalence of *A. cantonensis* infection in *Achatina fulica* in the nine municipalities that make up Baixada Santista, São Paulo, Brazil. **Methods**: *Angiostrongylus cantonensis* larvae were analyzed using optical microscopy. We performed polymerase chain reaction and restriction fragment length polymorphism using restriction endonuclease *ClaI*, directed to the internal transcribed spacer region 2 of *A. cantonensis* larval DNA. **Results**: Of the 540 snails analyzed, 117 (21.7%) were infected by *A. cantonensis*. For morphological and morphometric analyses, 60 larvae were used. Second-stage larvae were, on average, 358.2µm long and 26.4µm wide, while third-stage larvae were, on average, 450µm long and 21.12µm wide. The tails of the larvae ended in a fine tip. **Conclusions**: All municipalities comprising Baixada Santista had *A. fulica* that were naturally infected with *A. cantonensis*. All of the observed characteristics were typical of the species.

Keywords: Rat lungworm. Giant African snail. Eosinophilic meningitis. Nematode. Emerging parasitosis.

INTRODUCTION

Two of the 19 species from the *Angiostrongylus* genus can infect humans: *Angiostrongylus costaricensis* (Morera & Céspedes, 1971), which causes abdominal angiostrongyliasis[1] and *Angiostrongylus cantonensis* (Chen, 1935), which is the etiologic agent of eosinophilic meningoencephalitis, also called *rat lungworm*[2]. *A. cantonensis* has been observed in several regions of the world[3-7], and they were distributed from Eastern Asia to other continents by two main hosts: rats (definitive hosts) and *Achatina fulica* Bowdich, 1822 (one of the intermediate hosts), especially during the Second World War[8]. Several species of land and freshwater snails have also been found to be naturally infected with *A. cantonensis*[9-14].

In Brazil, the occurrence of *A. cantonensis* has been reported in all states except for Acre[9-17]. Man, being an accidental host, acquires parasitosis when eating foods contaminated with stage-three larvae (L3), raw or undercooked mollusks, and paratenic hosts such as shrimp, frogs, fish, and flatworms[4,18-20], as well as crabs and lizards[21,22]. In humans, these parasites migrate to the central nervous system (CNS), where they die in the meninges, causing inflammatory reactions[23 24].

Achatina fulica plays a crucial role in the global dispersion of *A. cantonensis*[1,8,22,25,26], since it is present in most areas where this nematode is endemic. These mollusks are associated with an anthropic environment, and once established, their population can significantly increase[27]. Remains of human activity favor the adaptation of this mollusk, as such remains provide food and shelter[28]. In Brazil, this mollusk has high potential to be involved in the transmission of *A. cantonensis* owing to its wide distribution, including to different ecosystems[29-31].

In the present study, the role of *A. fulica* as an intermediate host for *A. cantonensis* in the municipalities comprising Baixada Santista, São Paulo State, Brazil, was investigated.

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METHODS

Samples were collected from January to July, 2012. Specimens were captured in vacant lots in urban areas or where there were forest fragments or waste remains from 90 sites in the nine municipalities comprising Baixada Santista, São Paulo State: Bertioga, Cubatão, Guarujá, Itanhaém, Mongaguá, Santos, São Vicente, Peruíbe, and Praia Grande (**Figure 1**). Six adult snails were collected from ten sites in each municipality, for a total of 540 individuals. All 90 sites were characterized as to sanitary and georeferenced conditions. After identification of the snail, performed in accordance to Simone[32], the digestion procedure of mollusks was individually performed in accordance with methods of Wallace and Rosen[33], followed by the Baermann method[34]. *A. cantonensis* larvae were then counted and subjected to molecular analysis. The DNA was extracted from the pool of larvae from each snail using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The deoxyribonucleic acid (DNA) was subjected to polymerase chain reaction associated with restriction fragment length polymorphism (PCR-RFLP), and the primers used were directed to the internal transcribed spacer region 2 (ITS2) of ribossomal DNA (rDNA). NC1 (forward; 5'ACGTCTGGTTCAGGGTTGTT-3') and NC2 primers (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') were designed by Gasser[35] and anchored in the conserved regions in the final portion of subunit 5.8S and the initial portion of subunit 28S. Further, cleavage of this amplicon was performed with endonuclease *ClaI* (Biolabs) and the profiles were compared to those of *A. cantonensis* and *A. costaricensis* established by Caldeira[36]. For morphological and morphometric analysis, 60 larvae were used, which were fixed in 70% ethanol, clarified with Amann lactophenol, and analyzed (Leica Application Suite LAS V 3.8 Software and DMB 5000 Leica® microscope, Leica Microsystems, Wetzlar, Germany). The taxonomic identification of nematodes was based on morphological and morphometric parameters established by Ash[37] and Lv[10]. The SADIE index[38] was used to analyze the spatial patterns of the percentage of infected specimens from geographical coordinates and the percentage of infected *A. fulica*.

RESULTS

Achatina fulica was detected in anthropogenic environments, especially in those with great availability of food and shelter (82% of evaluated sites). Of the 90 sites analyzed, 73 (81.1%) had mollusks with nematode larvae, and, of these, 52 (71.2%)

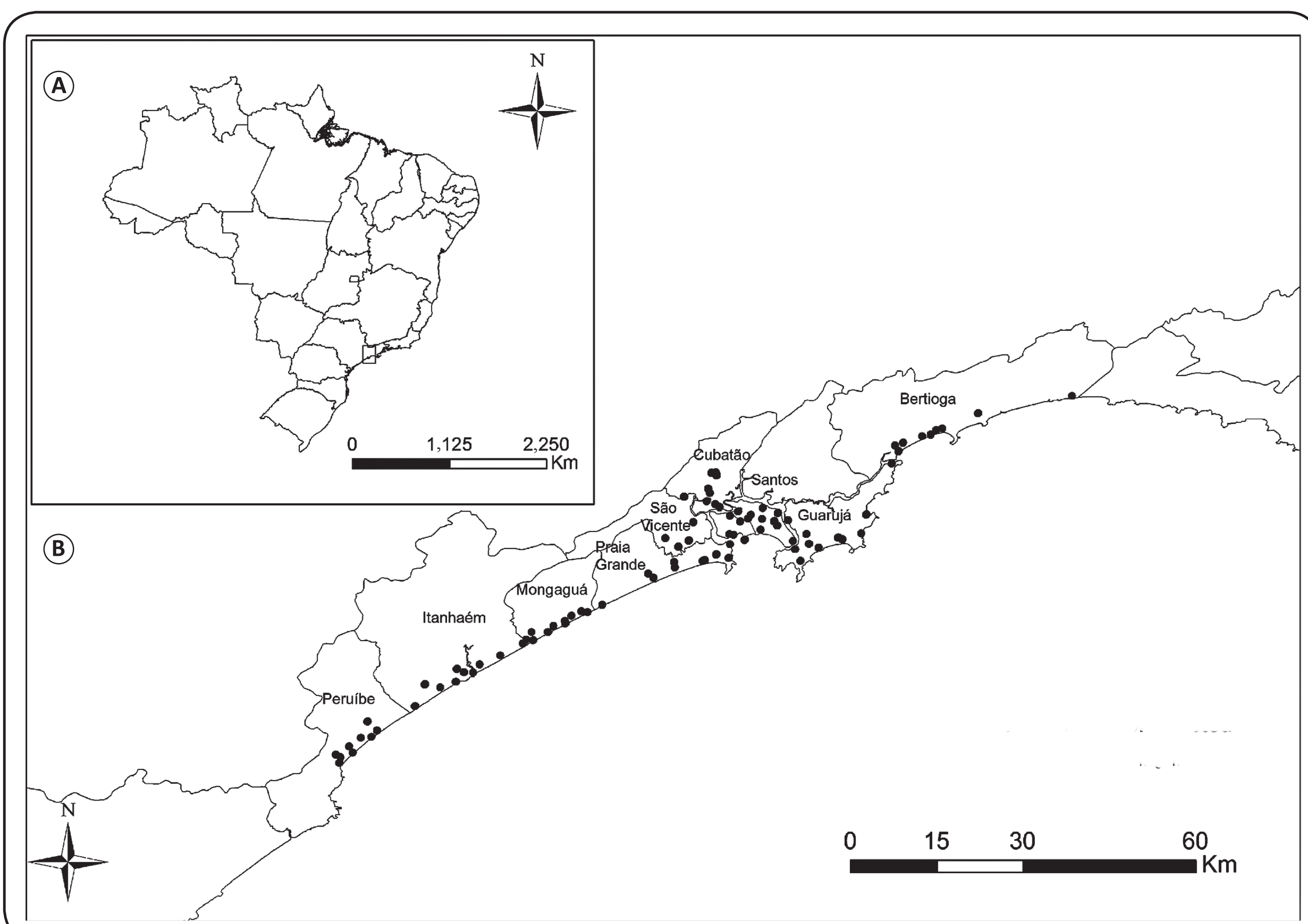

FIGURE 1 – A: Location of the study area and B: collection points in the municipalities comprising Baixada Santista, São Paulo, Brazil.

were infected with *A. cantonensis*. Of the 540 mollusks, 204 (37.7%) had nematode larvae, of which, 117 (57.3%) were infected with *A. cantonensis* (21.6% of the total) (**Table 1**). The prevalence of *A. cantonensis* infection in *A. fulica* for each municipality and the absolute number of parasite loads per mollusk are shown in **Table 2**.

The results were negative for the presence of *A. costaricensis*. Spatial analysis showed that the percentage of *A. fulica* infected with *A. cantonensis* in Baixada Santista had a random distribution, characterized by the absence of areas with much higher or much smaller infection percentages within the region (I = 1:38; p = 0.0957).

Morphological and morphometric analyses revealed that the larvae showed filiform bodies, striated cuticles in the transverse direction with rounded anterior ends showing two well-developed structures in the form of buttons and another in the form of a rod, followed by a long esophagus (**Figure 2**). The results of the morphological analyses of second-stage larvae (L2) and L3 of *A. cantonensis* are shown in **Table 3**.

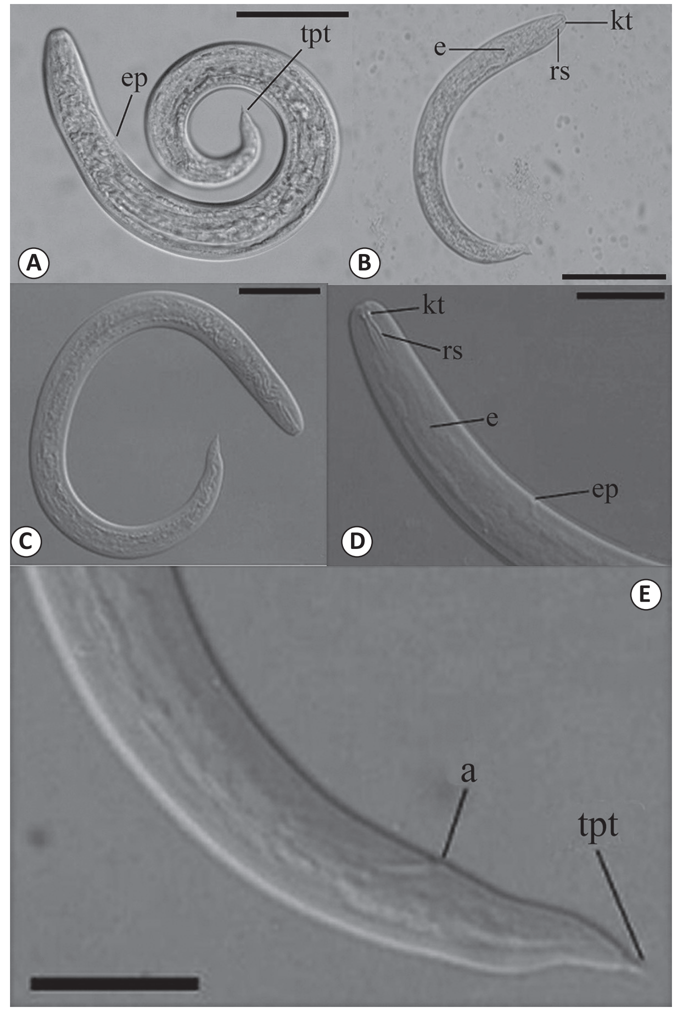

FIGURE 2 - ***Angiostrongylus cantonensis*** **isolated from** ***Achatina fulica*****. (A, B) Second-stage larvae (L2): scale, 50µm; (C, D) Third-stage larvae (L3): scale, 25µm; (E) Anterior end of L3 larvae showing anus and tail with pointed tip: scale, 25 µm. Legend: kt:** knob-like tips; **rs:** a rod-like structure; **e:** esophagus; **ep:** excretory pore. **Posterior end showing: tpt:** a tail with a pointed tip; **a:** anus.

DISCUSSION

Several snails play roles as intermediate hosts for *A. cantonensis*. Among them, the giant African snail *A. fulica* is one of the most important due to its abundance and occupation in different ecosystems. In this study, among 540 *A. fulica* specimens analyzed, 204 (37.8%) were found to contain nematodes, a value similar to that obtained by Rocco[39], who reported a rate of 34.2%. In both studies, specimens were obtained in anthropic environments where snails probably lived with small rodents, which is critical for the maintenance of parasites in the environment.

Recovered *A. cantonensis* larvae presented two morphotypes that were visually classified by morphometry and morphology as larval stages 2 and 3 (L2 and L3). Although the detail of the tail ending in a fine tip is a typical feature of the species, it cannot be used alone as a precise taxonomic identification factor[37]; however, L3 presented measures compatible with those obtained by Ash[37] and Thiengo[11] (**Table 3**).

Lv[10] found that, before the second molting, the main characteristics of L2 were similar to those of L3, as shown in **Figure 2**, which were two structures, similar to buttons and rods in shape. The founding of these two larval stages in the same snails is probably due to constant reinfections of the mollusk in the natural environment and to the method by which the analyzed material was obtained, in which the entire contents of the soft parts were processed.

Molecular analysis revealed the presence of *A. fulica* that were naturally infected with *A. cantonensis* in urban areas of the nine municipalities of the Baixada Santista region, with an infection rate of 21.7%. The variation of this rate is broad and has been observed in several municipalities, such as São Gonçalo (35.4%) and Barra do Piraí (10.3%), both in the State of Rio de Janeiro and Joinville/SC (27.4%)[12], China (13.4% and 28.4%)[40,41], Pernambuco (42%)[11], and Japan (52.79%)[42]. The climatic characteristics of Baixada Santista are appropriate for the development of *A. fulica* and *A. cantonensis*. In fact, Ishii[43] has observed that the L3 of *A. cantonensis* develop better at temperatures ranging from 20°C to 30°C. In addition to environmental factors such as temperature, variations in the infection rate can be influenced by biological cycle dynamics of the parasite in its hosts, by the population density of mollusks and rodents, and by biological characteristics[22,42,44].

These results indicate the need for more attention to this emerging parasite through awareness campaigns for local and medical communities, the development of a health surveillance system, improved health education, and the distribution of information about the management action adapted to each reality, since 82% of the analyzed wastelands had some type of garbage or rubble. Studies on the distribution of intermediate and paratenic hosts in areas near houses and the parasite-host compatibility should be investigated to improve understanding of transmission dynamics. In Brazil, there have been few

TABLE 1

Prevalence of nematode larvae and *Angiostrongylus cantonensis* in *Achatina fulica* mollusks in the nine municipalities comprising Baixada Santista, São Paulo, Brazil (n = 540; 60/municipality).

Municipality	**Number of *Achatina fulica* naturally infected with nematode larvae (%)**	**Number of *Achatina fulica* infected with *Angiostrongylus cantonensis* among those with nematode larvae (%)**
Bertioga	21/60 (35.0)	10/21 (47.6)
Cubatão	16/60 (26.7)	08/16 (50.0)
Guarujá	14/60 (23.3)	08/14 (57.1)
Itanhaém	28/60 (46.7)	15/28 (53.6)
Mongaguá	25/60 (41.7)	17/25 (68.0)
Peruíbe	34/60 (56.7)	15/34 (44.1)
Praia Grande	30/60 (50.0)	27/30 (90.0)
Santos	16/60 (26.7)	08/16 (50.0)
São Vicente	20/60 (33.0)	09/20 (45.0)
Total	**204 (37.7)**	**117/204 (57.3)**

TABLE 2

Prevalence of infection by *Angiostrongylus cantonensis* in *Achatina fulica* by each municipality and the absolute number of parasitic loads per snail.

Municipality	**Total of snails**	**Number of positive snails (%)**	**Individual parasitic load**
Bertioga	60	10 (16.7)	5; 7; 18; 36; 52; 98; 113; 148; 274; 9,723
Cubatão	60	8 (13.3)	4; 5; 17; 22; 30; 53; 82; 147
Guarujá	60	8 (13.3)	6; 11; 36; 187; 526; 703; 1,907; 2,407
Itanhaém	60	15 (25.0)	9; 19; 35; 36; 41; 42; 52; 61; 93; 109; 179; 215; 307; 601; 3,800
Mongaguá	60	17 (28.3)	6; 6; 7; 21; 21; 23; 30; 49; 62; 106; 110; 131; 349; 362; 448; 1,070; 3,213
Peruíbe	60	15 (25.0)	1; 3; 4; 4; 5; 8; 23; 27; 27; 66; 477; 937; 1,251; 1,302; 1,508
Praia Grande	60	27 (45.0)	1; 2; 3; 4; 11; 16; 19; 20; 28; 41; 45; 52; 54; 61; 74; 79; 85; 91; 126; 185; 203; 233; 388; 432; 568; 700; 1,717
Santos	60	8 (13.3)	1; 8; 12; 24; 281; 632; 1,328; 1,675
São Vicente	60	9 (15.0)	6; 14; 54; 61; 69; 160; 193; 285; 2,509

TABLE 3

Measurements (µm) of second- and third-stage larvae and tail characteristics of *Angiostrongylus cantonensis* retrieved from naturally infected *Achatina fulica*.

Characteristics	**L2**		**L3**	
	mean ± standard deviation	**variation**	**mean ± standard deviation**	**variation**
Body length	358.2 ± 27.8	299.5 - 399.2	450.8 ± 23.5	410.5 - 493.6
Width	26.4 ± 2.6	21.9 -34.5	21.1 ± 5.5	13.1 - 38.5
Esophagus length	145.2 ± 22.2	107.4 -236.0	168.7 ± 8.8	149.3 - 185.4
Excretory pore	61.9 ± 7.6	53.9 - 89.9	86.0 ± 4.3	77.9 - 93.2
Tail length	29.1 ± 3.4	21.2 - 39.7	35.3 ± 3.8	28.8 - 44.6
Termination of tail	Tapered		Tapered	

L2: second-stage larvae; **L3:** third-stage larvae.

studies on the action of *A. fulica* and other species of mollusks as intermediate hosts of *A. cantonensis* and their role in public health. For example, previous studies have shown the presence of other species naturally infected with *A. cantonensis* in addition to *A. fulica* in Brazil, such as *Bradybaena similares*, *Subulina octona*, *Sarasinula marginata,* and *Sarasinula linguaeformis*[9,11-14].

Most animal populations have aggregate spatial distribution patterns, generally owing to the distribution and supply of resources in the environment[45]. In this study, a regular spatial distribution pattern was observed, which is quite rare. The probability is that this distribution was due to the presence of high populations of *A. fulica* in urban areas related to their high adaptability, which makes it not a limiting resource of the *A. cantonensis* distribution. Furthermore, as the parasite can be found in different species of intermediate hosts, its spatial distribution becomes more regular. These data are especially useful and can be used by public health authorities to establish policies related to surveillance and planning of preventive actions. Isolated cases of eosinophilic meningoencephalitis have recently been reported in Brazil[9,11,14,15,46,47]. Thus, it is plausible that *A. cantonensis* continues to spread to new regions, increasing the risk of eosinophilic meningoencephalitis in humans.

Conflict of interest

The authors declare that there is no conflict of interest.

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